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Fresh water, marine and terrestrial cyanobacteria display distinct allergen characteristics

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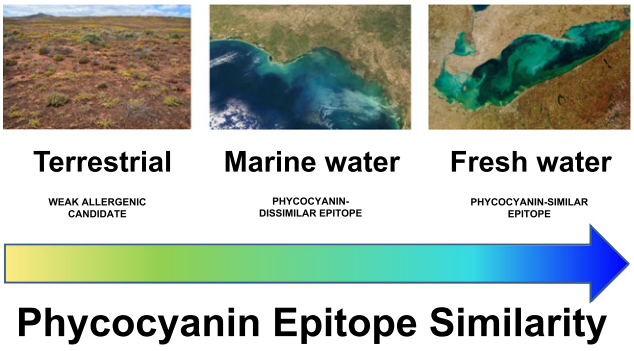
HIGHLIGHTS

- Cyanobacteria increase in abundance and usage, yet the allergenicity remained elusive.
- Allergenic potential of different taxa is demonstrated through three immunoassays.
- Similarities are observed between freshwater cyanobacteria and C-phycocyanin.
- *Nostoc* sp. shows anti-inflammatory and unique immune-reactive characteristics.
- Increase in public awareness would enable mitigation of the potential health risk.

GRAPHICAL ABSTRACT



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ABSTRACT

During the last decades, global cyanobacteria biomass increased due to climate change as well as industrial usage for production of biofuels and food supplements. Thus, there is a need for thorough characterization of their potential health risks, including allergenicity. We therefore aimed to identify and characterize similarities in allergenic potential of cyanobacteria originating from the major ecological environments. Different cyanobacterial taxa were tested for immunoreactivity with IgE from allergic donors and non-allergic controls using immunoblot and ELISA. Moreover, mediator release from human FcεR1-transfected rat basophilic leukemia (RBL) cells was measured, allowing in situ examination of the allergenic reaction. Phycocyanin content and IgE-binding potential were determined and inhibition assays performed to evaluate similarities in IgE-binding epitopes. Mass spectrometry analysis identified IgE-reactive bands ranging between 10 and 160 kDa as phycobiliprotein compounds. Levels of cyanobacterial antigen-specific IgE in plasma of allergic donors and mediator release from sensitized RBL cells were significantly higher compared to non-allergic controls ($p < 0.01$). Inhibition studies indicated

Abbreviation list: BSA, bovine serum albumin; C-PC, C-phycocyanin; DC, dendritic cell; HRP, horseradish peroxidase; FcεR1, human high-affinity receptor for IgE; KLH, keyhole limpet hemocyanin; LCM, linker core-membrane; LPS, lipopolysaccharides; PBS, phosphate buffered saline; PL, plasma; RBL, rat basophilic leukemia; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline + 0.05% Tween 20.

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Immunoassay
Inhibition assay

cross-reactivity between IgE-binding proteins from fresh water cyanobacteria and phycocyanin standard. We further addressed IgE-binding characteristics of marine water and soil-originated cyanobacteria. Altogether, our data suggest that the intensive use and the strong increase in cyanobacterial abundance due to climate change call for increasing awareness and further monitoring of their potential health hazards.

1. Introduction

Cyanobacteria are considered to be the most ancient photosynthetically-active life form, having played a key role in Earth history and the evolution of life on Earth (Knoll, 2008; Lenton and Daines, 2016). Thus they are found in all illuminated environments on this planet, including salty lakes, ice fields, hot springs, soils, fresh and marine waters (Whitton, 2012).

Various cyanobacteria serve as main biocatalysts in the nitrogen cycle (Issa et al., 2014), and they may be responsible for about 50% of global nitrogen fixation (Elbert et al., 2012). Some species are used for biofuel production, e.g., *Synechococcus* spp. (Nozzi et al., 2013), and others serve as food additives, e.g., *Arthrospira platensis* (*Spirulina*), *Nostoc* spp., *Synechocystis* spp. due to their high nutrient value (Borowitzka, 2013) and anti-inflammatory properties (Ku et al., 2013). Increased and expended marine cyanobacterial blooms have been reported due to environmental change, e.g., increase in temperature and CO_2 (Gobler et al., 2017), which may lead to an extended aerosolization of such species, carried toward the coastal areas (Lang-Yona et al., 2014).

Human exposure to cyanobacteria can occur through skin contact, consumption of food or food supplements, contaminated water, or inhalation of aerosolized cyanobacteria (Genitsaris et al., 2011; Bernstein and Safferman, 1970; Mittal et al., 1979; Schlichting, 1974; Sharma and Rai, 2008). Numerous cyanobacterial species can produce dermatotoxic, neurotoxic or hepatotoxic agents (van Apeldoorn et al., 2007), which may cause animal and, rarely, human deaths (Osborne et al., 2001). In addition to toxins, lipopolysaccharides (LPS) present in the outer membrane of cyanobacteria, have been reported to induce influenza-like illnesses (Annadotter et al., 2005) and were detected in air samples (Lang-Yona et al., 2014).

First reports on allergic reactions to cyanobacteria have appeared around 1950 (Heise, 1951), and since then, several studies demonstrated a correlation between cyanobacteria crude extracts and cutaneous hypersensitivities (Sharma and Rai, 2008; Bernstein et al., 2011; Stewart et al., 2006b; Torokne et al., 2001; Stewart et al., 2006a). Few case studies have reported severe allergic reactions upon exposure to cyanobacteria, i.e. anaphylactic shocks after food supplement consumption (Le et al., 2014; Petrus et al., 2010) and after swimming in a lake during cyanobacterial bloom (Geh et al., 2016).

Phycocyanin, a blue pigment protein complex, is commonly found in cyanobacteria and serves as light-absorbing compound in their photosynthetic apparatus (Schirmer et al., 1985). This pigment is widely used in the industry as a natural fluorescent dye, in food and beverages as a natural coloring agent, or in pharmaceutical applications (Kuddus et al., 2013). Recent studies have identified this compound as a potential allergen in two cyanobacteria species, i.e., *A. platensis* (Petrus et al., 2010) and *Microcystis aeruginosa* (Geh et al., 2015). However, a wider characterization of cyanobacterial allergens is still needed to comprehensively understand their allergenic potential, as well as similarities or differences of the allergens present in the different species.

We therefore aimed to analyze and characterize the allergic reactivity of seven cyanobacterial taxa originating from terrestrial, fresh and marine water environments. We compared the allergenic behavior of these species to the previously characterized allergenic cyanobacterium *M. aeruginosa* (Geh et al., 2015), and searched for similarities through protein identification and IgE-binding inhibition patterns.

2. Materials and methods

2.1. Cultures of cyanobacteria

Eight cyanobacterial taxa as listed in Table 1 were obtained from the Culture Collection of Algae at the Göttingen University (international acronym: SAG; Göttingen, Germany). Five species originating from fresh water (*Microcystis aeruginosa*, *Cylindrospermum siamensis*, *Anabaena ambigua*, *Lyngbya lagerheimii*, *Planktothrix agardhii*), two from marine (*Synechocystis* sp. and *Phormidium* sp.), and one was from a terrestrial environment (*Nostoc* sp.). Cultures were grown in different media as detailed in Table 1 at 20 °C, 120 rpm, and a 16 h/8 h light/dark cycle (76 μmoles m^{-2} s^{-1} daylight lamps; Heraeus BK 6160 low temperature incubator, Thermo Fisher Scientific, Darmstadt, Germany). Growing cultures were screened by light microscopy (Axio Imager A2, Zeiss, Göttingen, Germany) for purity of the cultures and morphological validation.

2.2. Human plasma samples

Six plasma samples (PL 1–6) were obtained from PlasmaLab Ltd. (Everett, WA, USA) and four (PL 7, PL N1–N3) from the Department of Dermatology (University Medical Center of the Johannes Gutenberg University, Mainz, Germany). Donors of plasma PL 1–7 were sensitized to a variety of aeroallergens and food allergens as summarized in Table 2, (the complete list is detailed in Table S1). Plasma N1–N3 were from non-allergic donors with no sensitization against aeroallergens or food allergens, with low levels of total IgE. All plasma samples were aliquoted and stored at −80 °C until use. The study was approved by the local ethics committee (Landesaerztekammer Rheinland-Pfalz, no. 837.055.16 (10374)), and written consent was obtained from all subjects in advance.

2.3. Protein extraction and quantification

Crude extraction of total proteins from cyanobacteria was performed as previously described (Ivleva and Golden, 2007) with some modifications (Lang-Yona et al., 2016). In brief, cultures of cyanobacteria in the late exponential growth phase were transferred into 50 mL tubes and centrifuged at 10,000 × *g* for 10 min. The pellets were washed with sterile phosphate buffered saline (PBS; Sigma-Aldrich, Munich, Germany), and resuspended in 200 μL PBS buffer before overnight storage at −80 °C. After thawing at 37 °C, the samples were placed on ice and a protease inhibitor cocktail (Sigma-Aldrich) together with 0.5 mm acid washed glass beads (Sigma-Aldrich) were added to the samples. They were then homogenized for 30 s using the Fastprep 24 homogenizer (MP Biomedicals, Heidelberg, Germany), and additional 100 μL PBS were added. After 10 min centrifugation at 4 °C and 10,000 × *g*, the supernatant was collected. This step was repeated twice followed by filtration through sterile 5 and 0.45 μm syringe filters (Pall Corporations, Bad Kreuznach, Germany). *Phleum pratense* and *Betula pendula* pollen (Allergon AB, Ängelholm, Sweden) were used as positive controls for the allergic reaction assays, and the extraction of proteins followed the same procedure as described above.

Protein concentrations were determined with Pierce bicinchoninic acid assay (BCA, Thermo Fisher Scientific) according to the

Table 1
Species tested for allergenic reaction.

Tested organism	Strain	Origin	Growth environment	Growth medium	Special characteristics	Reference
Microcystis aeruginosa	SAG 1450-1	Wisconsin, USA	Fresh water	ES[b]	Produce microcystin and cyanopeptolin	(Dittmann et al., 1997)
Cylindrospermum siamensis (Antarikanonda)	SAG 11.82	Thailand	Fresh water, damp soil	BG-11[a]	Grow under a wide range of environmental conditions	(Johansen et al., 2014)
Anabaena ambigua (C.B. Rao)	SAG 1403-7	India	Fresh water	ES[b]	Nitrogen fixing abilities; neurotoxin production	(Komárek, 2005)
Lyngbya lagerheimii (Gomont ex Gomont)	SAG 24.99	Hungary	Fresh water	BG-11[a]	Grow in mats; natural compounds used for pharmaceutics	(Komárek and Komárková-Legnerová, 1992)
Planktothrix agardhii (Gomont) Anagnostidis & Komárek	SAG 3.82	Norway	Fresh water	ES[b]	High abundance in shallow and eutrophic lakes; microcystin production	(Rücker et al., 1997)
Synechocystis sp. (C. Sauvageau)	SAG 37.92	California, USA	Marine water	1/2 SWES[b]	Used for biofuel production	(Lang et al., 2011)
Phormidium sp. (Kützing ex Gomont)	SAG 9.92	Spain	Marine water	SWES[b]	Red-pigmented cyanobacteria	(Komarek et al., 2014)
Nostoc sp. (Vaucher ex Bornet & Flahault)	SAG 70.79	France	Soil	ES[b]	Grow under a wide range of environmental conditions; characteristic morphology of colonies comprising filaments in a gelatinous sheaths	(Potts, 1997)
Phleum pratense	011312102	–	–	–		
Betula pendula	11101	–	–	–		

[a] Recipe from by Sammlung von Algenkulturen Göttingen (SAG) culture collection of algae.
[b] Recipes from SAG culture collection of algae.

manufacturer's protocol, and absorbance was measured with a microplate photometer (Multiskan GO, Thermo Fisher Scientific). The extracts were stored at −80 °C until further analysis.

2.4. SDS-PAGE and immunoblot

The extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% Precast Protein Gels (Mini-PROTEAN TGX, Bio-Rad, Munich, Germany) and detected with an imaging system (ChemiDoc XRS+, Bio-Rad). For Western blots, proteins were transferred onto PVDF membranes (PVDF Transfer Packs, Bio-Rad) using the Trans Blot Turbo semi-dry transfer system (Bio-Rad) according to the manufacturer's protocol. The membranes were washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T; Sigma-Aldrich), and blocked for 1 h with 5% bovine serum albumin (BSA; Sigma-Aldrich) in TBS-T buffer (5% BSA-TBS-T).

To detect human IgE-binding proteins, membranes were incubated overnight with 10% human plasma diluted in 5% BSA-TBS-T, followed by an overnight incubation with goat anti-human IgE antibody conjugated with horseradish peroxidase (HRP; Thermo Fisher Scientific) diluted in 5% BSA-TBS-T. To verify general reactivity of C-PC standard, detection was conducted with pooled plasma, containing PL 1, 3, 4 and 5. For the detection of phycocyanin compounds, blocked membranes were incubated overnight with a polyclonal rabbit anti-phycocyanin antibody conjugated to HRP (raised against C-phycocyanin whole protein from *Spirulina*, Biorbyt, Cambridge, UK) diluted in 5% BSA-TBS-T. Bound antibodies were developed with the enhanced chemiluminescence (ECL) Western Blotting Substrate kit according to the manufacturer's protocol (Pierce/Thermo Fisher Scientific). The resulting chemiluminescence was detected with the ChemiDoc XRS+ imaging system. Membranes were washed with TBS-T between all incubation steps and each assay was repeated 3 times.

2.5. IgE measurement and inhibition ELISA

The total and specific IgE concentrations were evaluated in human plasma as previously described (Bashir et al., 2013), with some modifications. Clear flat-bottom MaxiSorp 96-well plates (Nunc, Thermo Fisher Scientific) were coated with cyanobacteria/pollen extracts or C-phycocyanin from *Spirulina* sp. (C-PC; Sigma-Aldrich) at a protein concentration of 10 μg mL^{-1} in carbonate buffer. Wells reserved for standards and for the total IgE content were coated with anti-human IgE (A80-108A-15, Bethyl Laboratories, Inc., Montgomery, TX, US) at a concentration of 10 μg mL^{-1} in carbonate buffer. Plates were incubated at 4 °C overnight, washed three times with PBS-0.05% Tween 20 (PBS-T),

Table 2
Characterization of plasma samples for different allergens and total IgE levels.

Sample ID	IgE levels (kU L^{-1})										
	Phleum pratense	Other grass pollens	*Betula pendula*	Other tree pollen	Food	Weeds	Animals	Molds	Insects	Mites	Total[a]
PL 1	>100	>100	>100	>100	>100	33	40	37	24	81	5058
PL 2	90	90	37	43	29	10	92	61	12	32	5468
PL 3	>100	>100	12	32	32	38	3	21	26	2	5192
PL 4	78	[b]	>100	>100	>100	15	>100	65	[b]	>100	6036
PL 5	43	[b]	5	3	9	2	>100	10	1	>100	7085
PL 6	21	[b]	83	[b]	1	0.7	3	[c]	[d]	22	2391
PL 7	23.7	[d]	>100	[d]	68.1	[d]	[d]	[d]	[d]	[d]	2944
PL N1	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[d]	[c]	19
PL N2	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[d]	[c]	37.5
PL N3	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[d]	[c]	27

[a] Tested in the current study.
[b] Not detected.
[c] <0.35 kU L^{-1}.
[d] No information available.

and blocked with 1% BSA in PBS-T (1% BSA-PBS-T; Sigma-Aldrich) at room temperature for 1 h. Standard serial dilutions (0.975 to 250 ng mL^{-1}) of the human IgE calibrator (RC80-108-6, Bethyl Laboratories) in 1% BSA-PBS-T, as well as buffer blanks, were dispensed to the corresponding wells. Plasma samples and controls (diluted with 1% BSA-PBS-T) were added to the remaining wells and incubated at room temperature for 2 h. Bound IgE was detected with HRP conjugated goat anti-human IgE antibody (A80-108P-35, Bethyl Laboratories, Inc.; diluted 1:100,000 in 1% BSA-PBS-T) incubated at room temperature for 2 h. Color development was achieved in the dark by addition of 1-Step Ultra TMB-ELISA reagent (Thermo Fisher Scientific) and terminated with 2 mM sulfuric acid (Sigma-Aldrich) after sufficient color development. Absorbance was detected at 450 nm using a microplate photometer (Multiskan GO, Thermo Fisher Scientific). The amount of bound IgE antibody was calculated from the standard curves. Plates were washed with PBS-T between all steps and each assay was repeated 3 times.

ELISA inhibition assays were performed as described previously (de Leon et al., 2003) with some modifications. Briefly, 96-well polystyrene plates were coated with protein extracts (10 μg mL^{-1}) and blocked as described above. In order to obtain a better assessment of similarities in IgE-binding epitopes, pooled plasma (containing PL 1, 3, 4 and 5) and controls (diluted with 1% BSA-PBS-T) were pre-incubated with serial dilutions of the cyanobacterial extracts or, as a control, keyhole limpet hemocyanin (KLH; Sigma-Aldrich) in sterile 96-well conically shaped polypropylene plates (Nunc, Thermo Fisher Scientific) at room temperature for 2 h. One hundred microliters of the inhibition mixtures (including plasma with no inhibitor as positive control) were then dispensed into coated wells and incubated at room temperature for 2 h. The incubation with secondary antibody and color development was performed as described above. After correction for non-specific binding (control wells containing no antigen), percentage of inhibition was calculated using the following formula:

$$\%\text{inhibition} = 100 - \frac{OD_{405}\ \text{serum with inhibitor}}{OD_{405}\ \text{serum without inhibitor}} \times 100$$

2.6. Mediator release assay

The mediator release assay followed an established protocol (Ladics et al., 2008). In short, HuFcεRI-transfected rat basophilic leukemia (RBL) cells expressing the α-chain of human FcεRI were sensitized overnight with patients' plasma diluted in minimum essential medium containing 5% fetal calf serum (Biochrom, Berlin, Germany). After washing, cells were stimulated with serial dilutions of protein extracts in Tyrode's buffer containing 0.1% BSA and 50% D_2O. Degranulation was quantified by measuring the β-hexosaminidase activity in the culture supernatants by a substrate reaction with 4-Nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma-Aldrich). The absorbance was measured in a microplate reader at 405/620 nm, and the release was expressed as percent of the total cellular β-hexosaminidase content obtained by lysing the cells with Triton X-100 (Sigma-Aldrich) after correction for spontaneous release (cells incubated with plasma alone). Vitality of cells was evaluated before each step using light microscopy observation.

2.7. Statistical analysis

Data are reported as the mean ± SD. Significant differences between samples and controls were calculated using unpaired, two-sided Student's *t*-test, after normal distribution and homogeneity of variance were verified. Significance of inhibition levels between samples and negative control was evaluated using One Way ANOVA followed by Tukey post-hoc analysis for means comparison (Origin Pro 9). Levene's test for homogeneity of variance and normal distribution were verified prior to the analysis. Significant differences were assumed for $p < 0.05$.

3. Results

3.1. Immunoblot and protein analysis of cyanobacterial extracts

Cyanobacterial proteins were probed for IgE-binding using seven plasma samples from allergic patients (PL 1–7) and three samples from IgE-negative, non-allergic controls (PL N1–N3). Fig. 1A and B present protein bands and immunoblot results, respectively, for all eight cyanobacterial taxa, after reaction with PL 4 as a representative plasma. Cyanobacterial IgE-binding proteins were found in all species (Table S3), and the frequency of immunoblot bands varied for different plasma samples (Fig. S1). Strong bands were observed for *M. aeruginosa*, *C. siamensis*, *A. ambigua*, *L. lagerheimii*, *P. agardhii* and *Synechocystis* sp. (Fig. 1B, lanes 1–6 respectively). All fresh water cyanobacteria showed bands at ~100–110 kDa, whereas the marine taxa *Synechocystis* sp. and *Phormidium* sp. (Fig. 1B, lane 6 and 7) showed different IgE-binding protein patterns, with a common band detected at ~10 kDa. *Nostoc* sp. bands were relatively weak (Fig. 1B, lane 8) and resembled the fresh water band patterns. The control allergen, *P. pratense* grass pollen (Fig. 1B, lane 9), presented typical IgE-reactive bands of about 10, 13, 28, 34 and 50 kDa (Dolecek et al., 1993; Rajashankar et al., 2002; Suck et al., 2000). Mass spectrometry analysis (Table S2) revealed phycobilisome-related proteins in ~100 kDa bands, e.g., the phycobilisome rod-core linker and phycobilisome linker (both in the range of 30 kDa), phycobilisome linker core-membrane (LCM, 101.06 kDa) as well as phycocyanin β chain and phycobiliprotein core-membrane linker, ApcE (100.9 kDa), with the highest identification score. These bands were previously identified as IgE-binding proteins from *M. aeruginosa* and *Spirulina* (i.e., LCM protein and phycocyanin β subunit) (Petrus et al., 2010; Geh et al., 2015). Other high-score proteins varied between the different species and included ATPase, ATP synthase subunits, and Photosystem I and II proteins, with the latter being bound to the phycobilisome complex at the inner part of the chloroplast membrane (Sidler, 1995).

Fig. 1C shows the C-PC protein bands on SDS-PAGE (lane 1) compared to IgE-binding bands in immunoblot (line 2) from pooled plasma samples (PL 1, 3, 4 and 5). Prominent IgE-binding bands were detected at ~50 and 60 kDa. The four additional species presenting IgE-binding bands in the size range of C-PC (Fig. 1B) were *C. siamensis*, *L. lagerheimii*, *Nostoc* sp. and *Phormidium* sp. Proteins within the 50 kDa band belong to the phycobilisome complex as identified by mass spectrometry (Table S2, in bold), and the highest identification score for these proteins was given to *Phormidium* sp., in concordance with the observed bands in Fig. 1B.

Further characterization and quantification of the phycocyanin protein in the different cyanobacteria is presented in Fig. 1D. The phycocyanin-related bands in immunoblot can be divided into five groups: group I (160–250 kDa) includes phycocyanin hexamers (Morschel et al., 1980), group II (90–120 kDa) comprises the core-membrane linker polypeptides as well as phycocyanin trimers (Sidler, 1995; Morschel et al., 1980), group III (45–60 kDa) contains C-PC monomers (Morschel et al., 1980), group IV (30–40 kDa) represents the rod and rod-core linker polypeptides (Sidler, 1995), and group V (10–20 kDa) contains the monomeric form of phycocyanin (Bloomfield and Jennings, 1969), phycobiliprotein subunits and small linker proteins (Sidler, 1995). The bands expressed by each species are summarized and compared to the IgE-binding bands in Table S3. An overlap between phycocyanin and IgE-binding proteins occurred for one or more bands of each cyanobacterial taxon, as well as for the C-PC standard, where both bands detected by the phycocyanin antibody reacted with allergic donor IgE as well. The monomeric form of α- (~21 kDa) and β-phycocyanin-subunits (~18 kDa) did not react with the IgE-antibodies (Fig. 1C, lane 2). As expected, no phycocyanin-binding band was observed for the grass pollen control.

Phycocyanin bands of 100–110 kDa, indicating core-membrane linker polypeptides or the trimer form of C-PC (Fig. 1D, group II), and

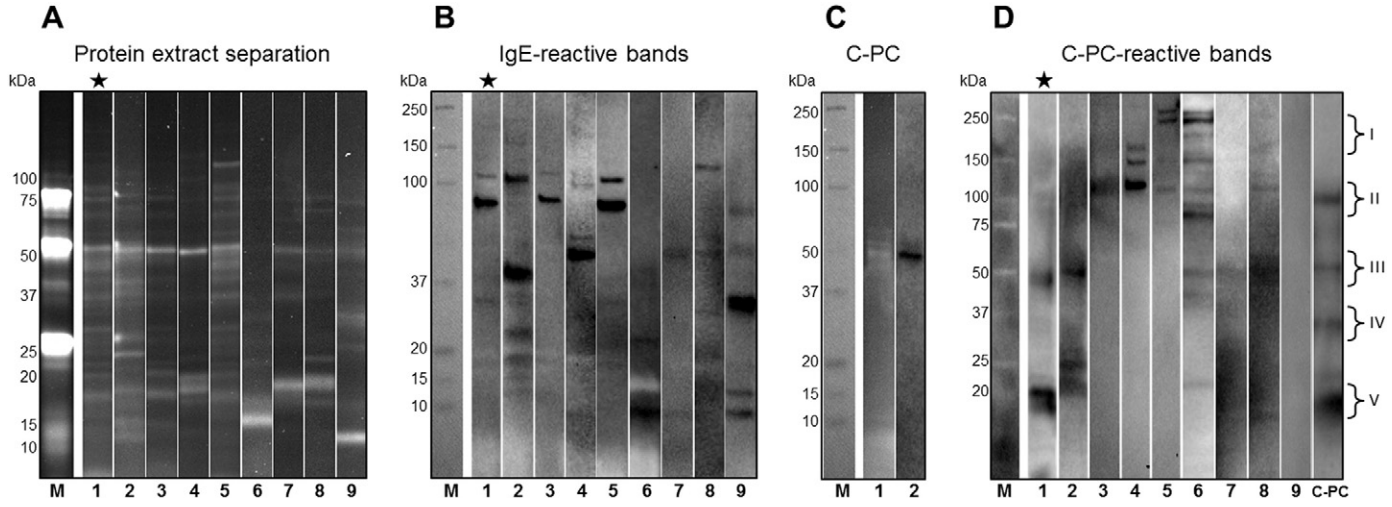

Fig. 1. Immunoblot analysis of cyanobacterial protein extracts. (A) SDS-PAGE separation of cyanobacterial protein extracts. (B) IgE-reactive bands from separated proteins blotted and probed with patient plasma. Results show species-extracts interacting with PL 4. (C) Immunoblot of C-PC standard with pooled patients' plasma (PL 1, 3, 4 and 5). Lanes: M, molecular weight standard; 1, C-PC protein bands; 2, IgE-binding protein bands. (D) C-PC-reactive bands from separated proteins blotted and probed with anti-phycocyanin antibody. Brackets I–V represent the five phycocyanin-related groups (Sidler, 1995; Morschel et al., 1980). Lanes in panel A, B and D: M, molecular weight standard; 1, *Microcystis aeruginosa*; 2, *Cylindrospermum siamensis*; 3, *Anabaena ambigua*; 4, *Lyngbya lagerheimii*; 5, *Planktothrix agardhii*; 6, *Synechocystis* sp.; 7, *Phormidium* sp.; 8, *Nostoc* sp.; 9, *Phleum pratense* and (D only) C-PC standard, respectively. Data are representative for ≥2 independent experimental repeats. The previously reported allergenic species *M. aeruginosa* is denoted (★).

corresponded with IgE-binding in fresh water cyanobacteria, and the *Nostoc* sp. (which presented weak phycocyanin-containing bands, superimposable to its IgE-binding bands, Fig. 1B, lane 9). Both *Synechocystis* sp. and *Phormidium* sp. showed distinct similarities between phycocyanin (Fig. 1D, lanes 6 and 7) and IgE-binding proteins, with multiple dual-reactive phycocyanin bands at higher molecular mass fractions for *Synechocystis* sp.

3.2. Higher cyanobacterial-specific IgE levels in plasma samples from allergic compared to non-allergic donors

To determine the amount of cyanobacteria specific IgE in the plasma samples, a quantitative ELISA was performed (Fig. 2). *Phleum pratense*-specific IgE levels are presented in comparison (Fig. 2, upper right). The different plasmas from the allergen-sensitized donors expressed varying amounts of IgE upon exposure to different cyanobacterial extracts. These IgE concentrations were approximately one order of magnitude lower than those obtained upon exposure to the *P. pratense* grass pollen extract (with levels over 100 kU L^{-1} in most plasmas), and in the same range as the phycocyanin-specific IgE. All examined donor plasmas presented significantly higher IgE levels compared to the negative control plasma, PL N1.

3.3. Induction of IgE-dependent and IgE-independent basophilic degranulation by cyanobacterial extracts

The presence of specific IgE levels in patients' plasma does not necessarily lead to IgE cross-linking and the initiation of an allergic response. Therefore, we further performed a mediator release assay with RBL cells expressing the human high-affinity receptor for IgE (FcεR1) on their surface (Vogel et al., 2005). In this assay, human IgE is bound by FcεR1, and functional cross-linking by allergens will lead to the release of mediators including β-hexosaminidase, which can easily be measured by a substrate reaction. Fig. 3 shows the results of a representative mediator release assay for cells sensitized with PL 4 and cross linked by exposure to serial dilutions of the different cyanobacterial extracts. High release levels were observed for *Nostoc* sp. (56 ± 3%), but similar levels were also detected after stimulation of the RBL cells sensitized with negative control plasma and treated with this cyanobacterial extract, and even without plasma sensitization.

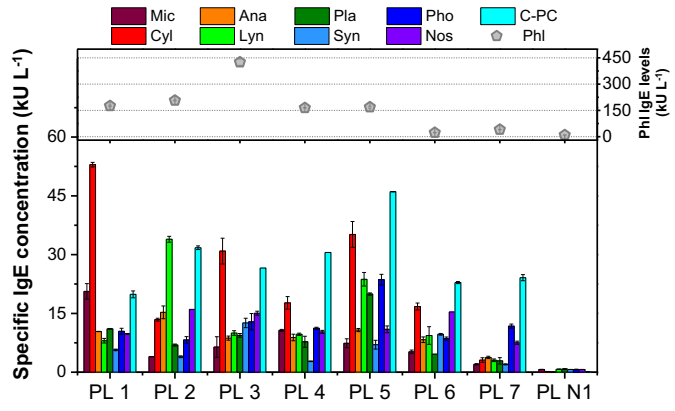

Fig. 2. Cyanobacteria-specific IgE concentration in examined plasmas. ELISA evaluation of specific IgE concentration against cyanobacteria and C-PC standard in plasma samples from allergic patients (PL 1–7) and a negative control plasma (PL N1). *Phleum pratense* grass pollen extract is shown for comparison. Data are representative of 3 independent experimental repeats.

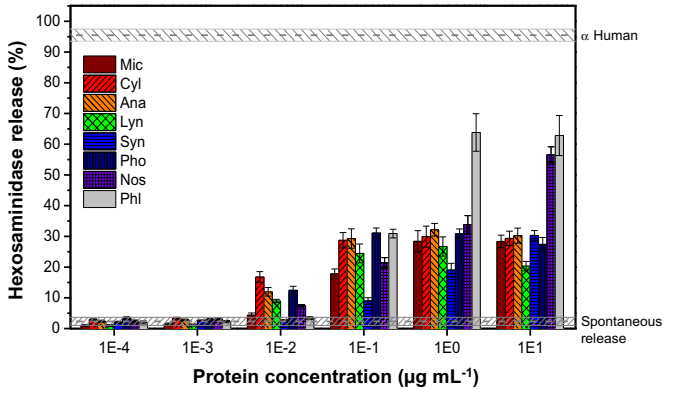

Fig. 3. In vitro characterization of cyanobacterial extracts. Mediator release from FcεRI-humanized RBL cells sensitized with human plasma (PL 4) was determined for serial dilutions of the cyanobacterial proteins. Anti-human IgE was used as positive control (upper dashed line), and cells without cyanobacterial extracts as negative control (lower dashed line). Mean values of triplicates are shown, with $p < 0.01$ compared with control plasma for all samples.

This indicates an IgE-independent degranulation response to antigens from this cyanobacterial species. The maximal release values of the other cyanobacteria did not differ much, and ranged from 26 to 31%. The species *C. siamensis* (30 ± 3%), *A. ambigua* (32 ± 2%), *L. lagerheimii* (27 ± 3%), and *M. aeruginosa* (28 ± 3%) showed their highest release levels at a protein concentration of 1 μg mL^{-1}. Mediator release induced by *Synechocystis* sp. (30 ± 2%) was highest at 10 μg mL^{-1} protein concentration, while *Phormidium* sp.-induced release was 31 ± 2% at 0.1 μg mL^{-1}. As a positive control, cells were sensitized with 0.3 μg mL^{-1} polyclonal human IgE and then cross-linked with goat anti-human IgE (1:500), or with PL 4 and timothy grass pollen extract, which resulted in maximal β-hexosaminidase release of 94 ± 5% and 64 ± 6% (at 1 μg mL^{-1}), respectively. No release was observed with the extracts for the control plasmas.

The unique behavior of *Nostoc* sp. was also noted in its anti-inflammatory properties (Fig. S1, A and B). This species was the only one showing a reduction of LPS-induced inflammation by 50% and 87% in the THP-1 cell line at 1 μg mL^{-1} and 10 μg mL^{-1}, respectively, compared to cells treated with LPS alone. Cyanobacteria-exposed THP-1 were tested for toxicity, as assessed by THP-1 viability demonstrating insignificant difference between THP-1 exposed to cyanobacterial extracts at both concentrations and the controls (see Supporting Information for more details).

3.4. Cross-reactivity between cyanobacterial species, pollen allergens and C-PC

To understand similarities in IgE-binding epitopes, we performed dose-dependent ELISA inhibition assays with cyanobacterial proteins as well as grass and birch pollen extracts (Fig. 4). The maximum inhibition values are presented in Fig. S2 as a heat map. Generally, *Nostoc* sp. and both the grass (*P. pratense*) and the birch pollen extract (*B. pendula*) were less efficient inhibitors for the tested cyanobacteria (compared to the average values), and pollen extracts were never exceeding an inhibition of 20% (Fig. 4, charts 1–8). Inhibition of *P. pratense* by cyanobacterial protein extracts was also relatively low (below 25% in all cases), whereas inhibition of *B. pendula* by cyanobacteria was higher, with highest rates observed by *Synechocystis* sp. (42.2 ± 4%), *A. ambigua* (38 ± 1%) and *P. agardhii* (34 ± 2%). Insignificant or no inhibition of IgE-binding was observed for the negative control (KLH). Self-inhibition in all samples was in the range of 70–80%.

Fresh water cyanobacterial extracts (Fig. 4, chart 1–5) showed cross-reactivity with *Spirulina*-extracted C-PC, which was significantly higher compared to species originating from other growth environments (Fig. 4, chart 6–8). Although low C-PC inhibited, marine species strongly inhibited fresh water cyanobacterial extracts, whereas fresh water species did not inhibit *Synechocystis* sp. or *Phormidium* sp.

4. Discussion

In recent years, cyanobacteria have been in the spotlight due to their beneficial effects, among which are their high nutrition levels and their ability to produce biofuel and assimilate carbon and nitrogen. As their global biomass also increases due to climate change, there is a need to properly characterize potential hazards associated with this phylum. In this study, we investigated the allergenic properties of cyanobacteria, thriving within different habitats and ecological niches.

We observed distinct IgE-binding patterns for the examined fresh water cyanobacteria. Analysis of the corresponding bands revealed phycobilisome-related proteins, previously identified as IgE-binding proteins from *M. aeruginosa* and *Spirulina* (i.e., LCM protein and phycocyanin β subunit) (Petrus et al., 2010; Geh et al., 2015). IgE binding was also found for the standard C-PC proteins, in contrast to the study by Geh et al. (Geh et al., 2015), where no IgE-reactive bands had been

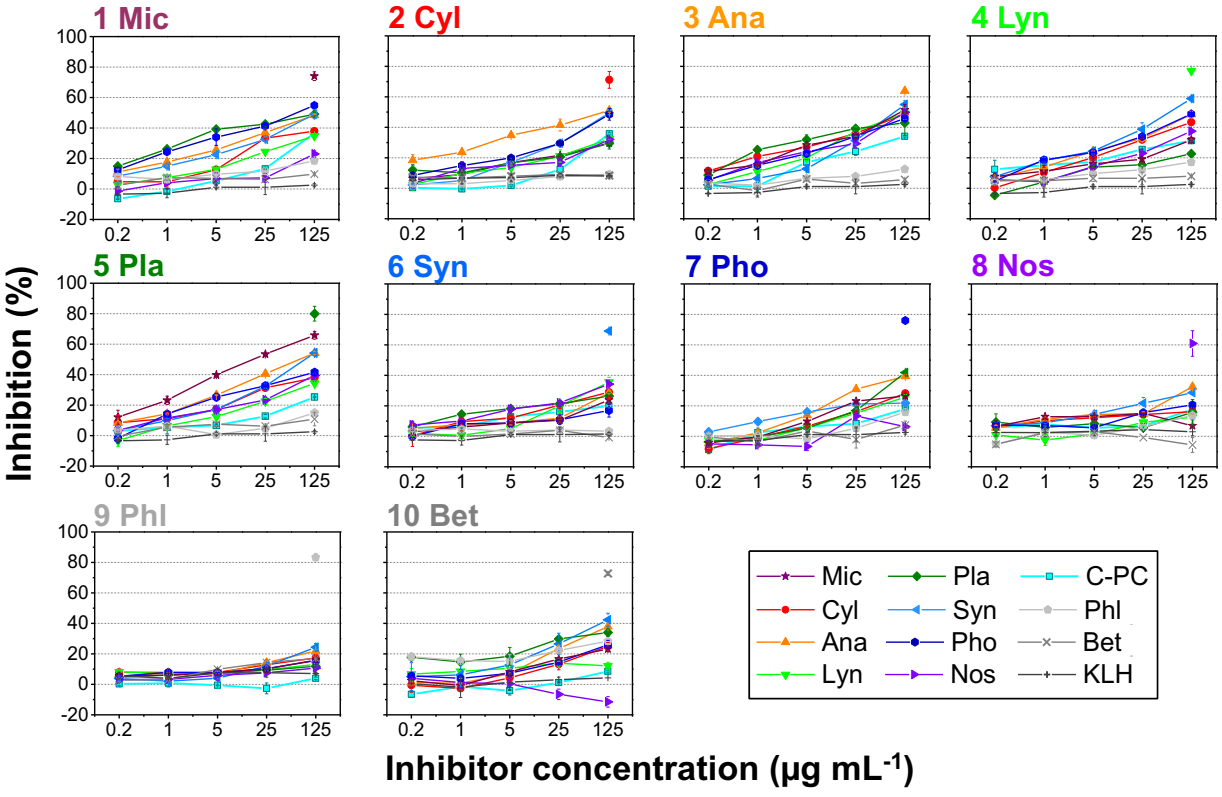

Fig. 4. Cross-reactivity of cyanobacterial proteins analyzed by inhibition ELISA. Cyanobacterial protein extracts (charts 1–8), *P. pratense* extract (chart 9), and *B. pendula* extract (chart 10) were tested for IgE-binding inhibition. Pooled plasma (including PL 1, 3, 4 and 5) were pre-incubated with cyanobacterial or pollen extracts and C-PC standard prior to their addition to plates, coated with extracts of the different cyanobacteria species as well as *P. pratense* and *B. pendula*, respectively. Bound IgE was quantified with an HRP-labeled anti-human IgE antibody. Data are representative of two independent experimental repeats. The same pooled plasma was used for all experiments.

detected for this standard. We attribute this difference to variations in specific IgE content in the tested plasmas/sera used in both studies. The absent IgE-binding signal of the monomer form of α- and β-phycocyanin subunits may indicate that the dimer form (~50 and 60 kDa) exposes IgE-binding epitopes different from the monomers.

The terrestrial *Nostoc* sp. presented weak IgE-binding bands, which might either result from low levels of IgE-binding proteins, lower exposure of the allergic individual to this subgroup or binding interferences by other substances. *Nostoc* spp. are known for their thick mucilaginous carbohydrate sheaths (Oren, 2014; Jordan, 2004), which might cause difficulties in isolation of different compounds from cells. They may also interact with specific compounds (e.g., proteins), as was previously shown for *Nostoc commune* DRH1 (Jordan, 2004), reducing their availability for IgE-binding. Nevertheless, the IgE-binding proteins shared common bands with phycocyanin in fresh water-derived cyanobacteria as well as *Nostoc* sp., suggesting the core-membrane linker polypeptides (indirectly binding the phycocyanin) or the trimer form of C-PC as IgE-binding candidates.

On the other hand, different IgE-binding bands were observed for *Synechocystis* sp. and *Phormidium* sp. (originating from marine environments), as well as distinct correlation patterns between phycocyanin and IgE-binding proteins. It might be that proteins other than phycocyanin are involved, or, alternatively, this may reflect differences in phycocyanin structure in these species. This conclusion is supported by previous publications that reported a unique phycocyanin structure of marine cyanobacteria (Sidler, 1995; Sonani et al., 2015). The differences in structure of the phycobiliprotein pigments compared to fresh water species are attributed to adaptation and modification for optimal light absorption in sea-water. However, conserved regions are yet present, allowing protein recognition by polyclonal antibody. The higher number of phycocyanin components as detected by western blotting using the anti-phycocyanin antibody compared to IgE-binding bands indicates a limited number of primarily allergenic proteins of the phycocyanin-complex.

In preliminary experiments C-PC was found to induce an upregulation of dendritic cell (DC) activation markers (data not shown), which was not due to LPS contamination. This implies that C-PC has intrinsic DC-activation properties that may contribute to its allergenicity, but this requires further investigation.

The different reactivity patterns of specific IgE against cyanobacterial strains indicate diverse epitope recognitions by IgE of the different plasma samples. This strengthens our statement that in addition to phycocyanin, other IgE-binding proteins/epitopes are present in cyanobacteria. The latter statement is further supported by the comparison of phycocyanin protein bands with the bands detected by patients' IgE, where some IgE-bound proteins were not identified as phycocyanin.

The false-positive behavior of the *Nostoc* sp. observed in the mediator release assay is most probably due to a substance that is able to activate the RBL cells independently from IgE, and not by an allergenic component. In a preliminary study, a slight degranulation could be observed for RBL cells, sensitized with various plasmas, cross-linked with C-PC standard. As the signal is not sufficient for the tested plasmas, to determine the C-PC allergenicity further, a larger cohort would be needed where the prior sensitization to cyanobacteria had been verified by skin tests.

The observed cross-reactivity of fresh water cyanobacteria with *Spirulina*-extracted C-PC may be explained by the origin of the *Spirulina*, which is mostly grown in fresh water (Vonshak, 1997). Thus, the structures of the phycocyanins probably share similarities in IgE-binding epitopes. Marine species strongly inhibited fresh water cyanobacterial extracts, however, a mild inhibition for the marine species was observed by C-PC. These results emphasize structural differences in phycocyanin molecules between species (Sidler, 1995), which apparently affect their IgE-binding efficiencies. Moreover, other molecules may participate in IgE-binding and in the potential induction of allergies by cyanobacteria.

The moderate to high inhibition of *B. pendula* by cyanobacterial strains implies a potential similarity between the cyanobacterial IgE-binding proteins and a selected *B. pendula* protein, requiring further investigation. The insignificant inhibition of *B. pendula* by phycocyanin suggests an alternative cyanobacterial IgE-binding protein for the observed similarity.

The low IgE-binding abilities observed for the *Nostoc* sp. throughout all assays, together with the false-positive stimulation of IgE cross-linking lead to the conclusion that this taxon is an unsuitable candidate for allergy induction. Self-inhibition of cyanobacteria is most probably influenced by other compounds in the crude protein extracts which interfere with IgE-binding, reducing binding efficiency to epitopes (Chlipala et al., 2009).

In conclusion, we observed distinguished allergenic activity of the examined fresh water cyanobacteria compared to marine- and soil-derived taxa. The standard C-PC from *Spirulina* demonstrated similarities in all immunoassays for fresh water cyanobacteria, supporting its role as a prime allergenic protein candidate in these species. Differences in IgE-binding proteins compared to the phycocyanin protein patterns observed in immunoblot and inhibition assays, indicate the presence of additional allergenic proteins, which should be investigated in further detail. Differences in inhibition patterns and in immunoassays between fresh water species and cyanobacteria from other environments suggest different allergenic protein types or structures. Low allergenic potential was determined by combined analyses for the terrestrial *Nostoc* sp., and the involvement of its mucilaginous layer in inhibition of the protein activity and availability should be examined. Further studies are needed to identify and fully characterize these allergens, including the abundance of such allergies in the general population, the suspected additional allergenic proteins observed in this study and the similarities with other allergens.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.08.069.

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